JOURNAL OF VIROLOGY, Nov. 1997, p. 8475–8481 Vol. 71, No. 11
0022-538X/97/$04.00+0

Proteolytic Activation of Tick-Borne Encephalitis Virus by Furin

KONRAD STADLER, STEVEN L. ALLISON,* JULIANE SCHALICH, AND FRANZ X. HEINZ

Institute of Virology, University of Vienna, A-1095 Vienna, Austria

Received 19 May 1997/Accepted 1 August 1997

Flaviviruses are assembled intracellularly in an immature form containing heterodimers of two envelope proteins, E and prM. Shortly before the virion exits the cell, prM is cleaved by a cellular enzyme, and this processing step can be blocked by treatment with agents that raise the pH of exocytic compartments. We carried out in vivo and in vitro studies with tick-borne encephalitis (TBE) virus to investigate the possible role of furin in this process as well as the functional consequences of prM cleavage. We found that prM in immature virions can be correctly cleaved in vitro by recombinant bovine furin but that efficient cleavage occurs only after exposure of the virion to mildly acidic pH. The data suggest that exposure to an acidic environment induces an irreversible structural change that renders the cleavage site accessible to the enzyme. Cleavage by furin in vitro resulted in biological activation, as shown by a 100-fold increase in specific infectivity, the acquisition of membrane fusion and hemagglutination activity, and the ability of the envelope proteins to undergo low-pH-induced structural rearrangements characteristic of mature virions. In vivo, prM cleavage was blocked by a furin inhibitor, and infection of the furin-deficient cell line LoVo yielded only immature virions, suggesting that furin is essential for cleavage activation of flaviviruses.

The surface glycoproteins of many enveloped viruses are initially synthesized as inactive precursors, and proteolytic cleavage is often required for maturation and full functional activity. In several virus families, this processing step is carried out by cellular proprotein convertases (reviewed in reference 16), most commonly furin, a component of the constitutive secretory pathway of many different types of cells (7, 35). Furin is a membrane-bound, calcium-dependent subtilisin-like protease whose primary site of action is the *trans*-Golgi network (TGN), although cycling of furin between the exocytic and endocytic pathways and the plasma membrane has also been demonstrated (4, 21). This enzyme is also secreted from cells in an active soluble form which is produced by self-cleavage in the TGN (39, 41).

In some cases (e.g., orthomyxoviruses, retroviruses, and paramyxoviruses), proteolytic cleavage of the viral fusion protein precursor liberates a stretch of amino acids, located at the amino terminus of the membrane-associated part of the polypeptide, which is required for fusion activity (the fusion peptide) (reviewed in reference 46). In other cases, the fusion protein is held in an inactive state by its association with a second protein in a heterooligomeric complex and it is the cleavage of the second protein that activates the fusion potential of the virus. This type of activation has been demonstrated with alphaviruses (18, 29, 44), and there is indirect evidence that flavivirus activation occurs in an analogous manner (8, 9, 12, 27, 34, 45).

The flaviviruses (genus *Flavivirus*, family *Flaviviridae*), whose members include arthropod-borne human pathogens such as yellow fever (YF) virus, Japanese encephalitis (JE) virus, the dengue viruses, and tick-borne encephalitis (TBE) virus, are small enveloped viruses with a positive-stranded RNA genome. The mature form of the flavivirus envelope contains two transmembrane proteins, one of which, the envelope protein E, is believed to carry out the dual functions of binding cell surface receptors and then, after uptake by endocytosis, mediating the fusion of viral and endosomal membranes (22, 28). Fusion is apparently initiated by a low-pH-induced rearrangement which results in the quantitative conversion of metastable homodimeric E proteins to a trimeric form (1).

Newly synthesized E proteins, however, are not homodimeric but first form stable heterodimeric complexes with the viral glycoprotein prM (2, 45). Assembly of these prM-E heterodimers with the nucleocapsid leads to the formation of immature virions, which are transported through the endoplasmic reticulum, Golgi, and TGN. Shortly before the virus is released from the cell, a cellular protease cleaves off slightly more than half of the amino-terminal end of prM and leaves the C-terminal portion (mature M) anchored in the membrane (5). This cleavage occurs immediately after the amino acid sequence Arg-X-Arg/Lys-Arg (X is variable) (5), which corresponds to the consensus sequence for furin (16). Earlier studies (8, 9, 12, 27) have shown that prM cleavage can be blocked in vivo by raising the pH in exocytic compartments, but the mechanism of this inhibition is still not understood.

Studies with immature virions have shown that they are less infectious than the mature form (8, 12, 27, 34, 45) and, unlike mature virions, are incapable of inducing cell-cell fusion (8, 9) or hemagglutination (12, 32) at low pH. Immature virions also fail to undergo the structural rearrangements believed to be required for these processes (1, 12, 32), suggesting that the low-pH-induced reorganization of E proteins in the viral envelope is physically blocked by the presence of prM.

In this study, we investigated the role of proteolytic cleavage of prM in the biological activation of TBE virus and the involvement of furin in this process. We demonstrate that biological properties such as hemagglutination activity, fusogenicity, and infectivity can be activated by in vitro cleavage of prM with recombinant bovine furin and that an irreversible low-pH-induced structural change in the immature virion is apparently needed before cleavage can occur. Experiments with a furin-deficient cell line and a specific inhibitor of furin suggest that

* Corresponding author. Mailing address: Institute of Virology, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria. Phone: 43-1-404-90, ext. 605. Fax: 43-1-406-21-61. E-mail: steven.allison@univie.ac.at.

this protease is required for the in vivo maturation of TBE virus.

MATERIALS AND METHODS

Cells and virus. BHK-21 (Baby hamster kidney) cells were grown in minimal essential medium supplemented with 5% fetal bovine serum and 1% neomycin at 37°C in an atmosphere of 5% CO_2. LoVo cells were purchased from the American Type Culture Collection and grown in Ham's F12 medium supplemented with 20% fetal bovine serum at 37°C in 5% CO_2. The cells were infected at a multiplicity of infection (MOI) of 1 with TBE virus Western subtype prototype strain Neudoerfl (19), with an infected baby mouse brain suspension as the inoculum.

Virus for in vitro experiments was grown in primary chicken embryo cells and purified by rate-zonal and equilibrium centrifugation in sucrose gradients as described previously (11). Immature virions were produced in the same manner as mature virions, except that the cells were treated with 20 mM ammonium chloride to raise the pH of exocytic compartments and thereby prevent prM cleavage (12). Virions released from these cells had therefore not yet been exposed to an acidic environment.

Furin-specific inhibitor. Decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (dec-RVKR-CMK) (40) was generously provided by Wolfgang Garten and Hans-Dieter Klenk, Institute of Virology, University of Marburg, Marburg, Germany. The inhibitor was dissolved in dimethyl sulfoxide at a concentration of 10 mM, and this stock solution was added directly to the cell culture medium.

Gel electrophoresis and immunoblotting. Proteins from cell supernatants or furin-treated virus samples were precipitated with deoxycholate and trichloroacetic acid and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels made with 15% acrylamide (17). For immunodetection of TBE virus proteins, a Bio-Rad Trans-blot semidry transfer cell was used to blot proteins from the gel onto polyvinylidene difluoride (PVDF) membranes. The membranes were first incubated at room temperature in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 and 1% bovine serum albumin to block nonspecific binding and then incubated for 60 min at room temperature with a 1,000-fold dilution of a polyclonal antiserum from a rabbit that had been immunized with whole purified TBE virus. This antiserum was chosen for its ability to detect the proteins prM and M as well as E. The membranes were then washed three times, and the proteins were visualized with peroxidase-labeled donkey anti-rabbit immunoglobulin and 3,3′-diaminobenzidine-H_2O_2 and $NiCl_2$ as specified by the manufacturer (Sigma).

Protein sequencing. Proteins were separated by SDS-PAGE and transferred to PVDF membranes as described above. The protein bands were then stained with Coomassie blue R-250 (Bio-Rad). Automated N-terminal protein sequencing was carried out by R. Prohaska at the Institute of Biochemistry, University of Vienna, Vienna, Austria, with an Applied Biosystems 477A sequencer.

Production of r-furin. Recombinant bovine furin (r-furin) was produced in COS-1 cells (ATCC CRL 1650) by transfection with the expression plasmid pSG5:bfur (31), which was kindly provided by Martin Vey, Institute of Virology, University of Marburg. One day after transfection the cell culture medium was replaced by serum-free HEPES-buffered Dulbecco's minimal essential medium (Life Technologies), and the supernatant containing secreted r-furin was harvested 2 days later. The supernatant was cleared by centrifugation (30 min at 4°C and 10,000 rpm in a Sorvall RC5C centrifuge) and concentrated approximately 100-fold by ultrafiltration in a Centriprep 30 concentrator (Amicon). This material was then layered onto a 5 to 20% sucrose gradient containing 50 mM Tris-maleate (pH 7.4) and 10 mM $CaCl_2$ and centrifuged overnight at 38,000 rpm in a Beckman SW-40 rotor at 4°C. Fractions of 0.6 ml were collected, and a protease inhibitor cocktail was added to each fraction to yield the following final concentrations: leupeptin, 10 μM; pepstatin A, 0.4 mM; *trans*-epoxysuccinyl-1-leucylamido(4-guanidino)butane, 10 μM.

The furin activity was determined fluorometrically as described by Stieneke-Gröber et al. (36), with *N*-α-*t*-butyloxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin (boc-RVRR-AMC; purchased from Bachem Feinchemikalien AG, Bubendorf, Switzerland) as the substrate. Assays were carried out at 37°C at a standard substrate concentration of 10 μM in a reaction buffer of 50 mM Tris-maleate (pH 7.4)–10 mM $CaCl_2$. One unit was defined as the amount of furin required to cleave 1 nmol of substrate in 1 min under the assay conditions.

In vitro activation of immature virions. "In-vitro-activated" virions for functional studies were produced by incubation of purified immature virions at 1 μg/ml for 10 min at 37°C in a buffer containing 50 mM Tris-maleate (pH 6.2) and 10 mM $CaCl_2$, followed by addition of 4 volumes of the same buffer adjusted to pH 7.4. Furin (30 to 100 U) was then added, and incubation at 37°C was continued for 2 h.

Infectivity, hemagglutination, and fusion assays. For infectivity measurements, the virus titer was determined on porcine kidney (PS) cells at 37°C by the plaque assay described by Holzmann et al. (13). Hemagglutination activity was measured at pH 6.4 with goose erythrocytes as described by Clarke and Casals (6).

TBE virus-induced cell-cell fusion activity was tested with C6/36 mosquito cells in a fusion-from-without assay system as described by Schalich et al. (32). Virus samples were tested at a standard concentration of 300 μg/ml, and the fusion reaction was carried out at pH 5.5 with morpholineethanesulfonic acid (MES)-buffered culture medium.

Sedimentation analysis. The oligomeric state of E proteins in the virion envelope was determined by solubilization of virions in 0.5% Triton X-100 and sedimentation in 7 to 20% sucrose gradients containing 0.1% Triton X-100 as described by Allison et al. (1). Protein E in the gradient fractions was quantitated by four-layer enzyme-linked immunosorbent assay after denaturation with SDS as described by Heinz et al. (12).

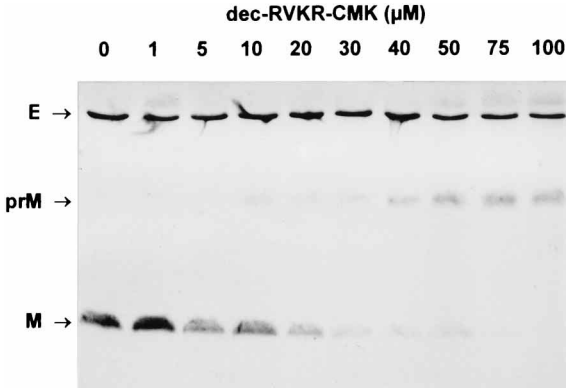

FIG. 1. Inhibition of prM cleavage by dec-RVKR-CMK. Monolayers of BHK-21 cells were treated with the inhibitor at the indicated concentrations and infected with TBE virus. Proteins from the secreted virions were analyzed by SDS-PAGE and immunoblotting 24 h after infection. The positions of the E, prM, and M protein bands are shown.

RESULTS

prM cleavage is blocked in vivo by a furin-specific inhibitor. To investigate whether furin is the host cell protease responsible for the cleavage of the TBE virus prM protein, BHK-21 cells, which contain endogenous furin (30), were treated 1 h before infection with different concentrations of dec-RVKR-CMK, a peptidyl chloromethylketone derivative which has been shown to inhibit furin in vivo (40). Virus from cell supernatants was analyzed 24 h postinfection by SDS-PAGE and immunoblotting with a rabbit anti-TBE virus antiserum recognizing the proteins prM, M, and E. As shown in Fig. 1, prM cleavage was efficient in untreated BHK-21 cells, and nearly all of the virus released from the cells was in the mature form. Treatment with dec-RVKR-CMK, however, resulted in the release of virions containing uncleaved prM, and essentially complete inhibition was observed at inhibitor concentrations of 75 μM or above.

prM is not cleaved in furin-deficient cells. To obtain more evidence that furin itself, rather than a closely related protease, is responsible for prM cleavage, LoVo cells, which have a specific genetic defect causing them to lack functional furin (37, 38), were infected with TBE virus at an MOI of 10 and the protein composition of virions released from these cells 48 h after infection was analyzed by SDS-PAGE and immunoblotting. As a control, virions produced by infection of BHK-21 cells at the same MOI were also analyzed. As shown in Fig. 2, the TBE virions secreted from LoVo cells, in contrast to those from BHK-21 cells, were still in the immature form and showed no detectable M protein. These results, together with those from the previous experiment, provide strong evidence that furin is essential for prM cleavage.

The immunoblot of the BHK-21 cell control in Fig. 2 contained an additional band with an apparent molecular weight of about 17,000. This band was also found in subsequent immunoblotting experiments, but only in samples containing ma-

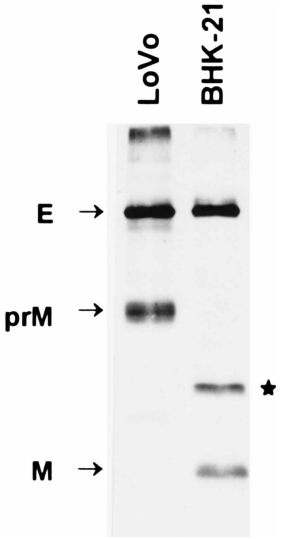

FIG. 2. SDS-PAGE and immunoblot analysis of TBE virus grown in LoVo and BHK-21 cells. Cells were infected at an MOI of 10, and virus was harvested 48 h after infection. Before analysis, the virus was partially purified by sucrose gradient centrifugation. The positions of the E, prM, and M protein bands are labeled, and the position of the M dimer band (see the text) is indicated by a star.

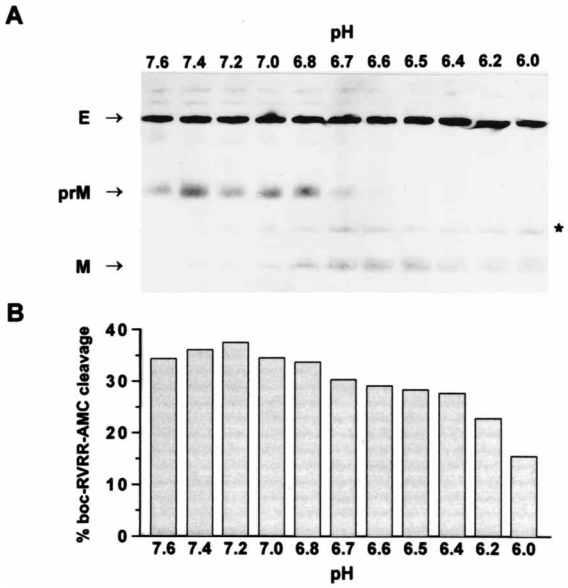

FIG. 3. pH dependence of in vitro cleavage of immature virions and boc-RVRR-AMC by r-furin. (A) Immature virions were incubated at 37°C for 2 h at the indicated pH value with 30 U of r-furin and analyzed by SDS-PAGE and immunoblotting. (B) The artificial substrate boc-RVRR-AMC was cleaved at the same pH values (see Materials and Methods).

ture M and not in immature virion preparations (see also Fig. 3A and 4). It was also present when purified virions from primary chicken embryo cell supernatants were used and was detectable with several different TBE virus-specific antisera (data not shown). To identify this band, its N-terminal sequence was determined by Edman degradation after blotting of purified envelope protein preparations onto a PVDF membrane (see Materials and Methods). The experimentally determined sequence XVLIPSHA (X is an unidentified residue) corresponded to the N-terminal sequence of the M protein, SVLIPSHA (19). We therefore concluded that this band represents an SDS-resistant oligomer of M, probably a dimer, which reacts especially strongly with the antiserum, since it appears to be present in low abundance in Coomassie blue-stained gels (data not shown).

Recombinant furin cleaves prM in vitro at acidic pH. It has been shown previously that prM cleavage in flavivirus-infected cells can be suppressed by agents that raise the pH in acidic cellular compartments (8, 9, 12, 27, 32), suggesting that efficient processing of prM by furin occurs only under acidic conditions. We therefore carried out in vitro experiments for investigating the pH dependence of prM cleavage by using a soluble form of bovine furin (r-furin) (31) produced by expression of the cloned gene in COS-1 cells (see Materials and Methods).

Purified immature virions at 1 μg/ml were incubated for 2 h at 37°C with 30 U of r-furin (see Materials and Methods for definition of a unit) at the pH values indicated in Fig. 3 and analyzed by gel electrophoresis and immunoblotting. The data presented in the figure clearly show that prM cleavage was pH dependent, with a sharp increase in efficiency below pH 6.7. This, however, does not reflect the pH dependence of the activity of r-furin itself, which, consistent with earlier reports (20, 36), was active over a broad range with a slight optimum around pH 7.3 when assayed with the artificial substrate, boc-RVRR-amidomethylcoumarin (Fig. 3B). The data indicate, rather, that the observed pH dependence was due instead to changes in the cleavability of prM itself.

The N-terminal portion of prM (pr), which was removed by protease cleavage and whose electrophoretic mobility is similar to but distinct from that of the M dimer described above, was not visible in the immunoblots but could be detected by Coomassie blue staining. Its identity was confirmed by direct N-terminal protein sequencing (data not shown).

The pH dependence of prM cleavability is apparently due to an irreversible change in the immature virion. To investigate the reversibility of the low-pH-induced changes necessary for prM cleavage, we exposed immature virions to pH 6.2 for 10 min and then back-neutralized them to pH 7.4 before adding r-furin (Fig. 4). The cleavage pattern was compared to those of controls that had been treated at pH 6.2 or 7.4 only. The controls in lanes 2 and 3 show that, as in the previous experiment, prM was efficiently cleaved at pH 6.2 but not at pH 7.4. However, prM in virions that had been preincubated at pH 6.2 and then back-neutralized could now be cleaved at pH 7.4 as

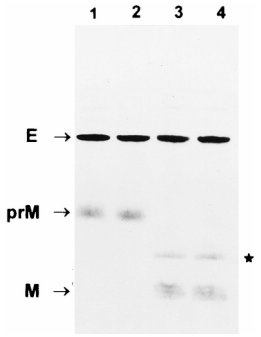

FIG. 4. In vitro cleavage of prM in immature virions after low-pH treatment. Proteins were analyzed by SDS-PAGE and immunoblotting after immature virions were treated as follows: 2-h incubation at pH 7.4 without r-furin (lane 1), 2-h incubation with r-furin at pH 7.4 (lane 2), 2-h incubation with r-furin at pH 6.2 (lane 3), and 2-h incubation with r-furin at pH 7.4 after a 10-min preincubation at pH 6.2 (lane 4). The positions of the E, prM, and M bands are labeled, and the position of the M dimer band is indicated by a star.

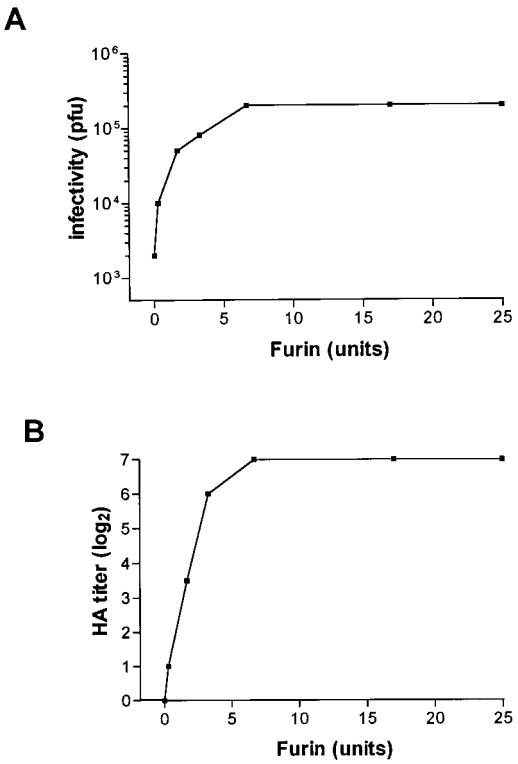

FIG. 5. Biological activation of immature virions by furin treatment. After incubation for 10 min at pH 6.2 and readjustment to pH 7.4, immature virions were treated with different amounts of r-furin and tested for infectivity by a plaque assay (A) and for HA activity (B).

well (lane 4), suggesting that low pH induces an irreversible structural change that causes the cleavage site in prM to remain accessible to the enzyme even after back-neutralization.

prM cleavage in vitro activates biological functions. Since immature virions containing uncleaved prM lack HA activity and are less infectious than mature virions, it was predicted that an increase in these activities should be observable after cleavage of prM in vitro. To test this, purified immature virions at 1 μg/ml were preincubated at pH 6.2 for 10 min, back-neutralized to pH 7.4, and then treated for 2 h at 37°C with different amounts of r-furin. The infectivity and HA titers of each of these samples were then determined and compared to those of untreated controls. As shown in Fig. 5A, increasing the amount of furin in the range of 0 to 7 U led to an approximately 100-fold increase in specific infectivity. No further increase was observed when more r-furin was added, consistent with earlier observations in which similarly prepared immature virions were not found to be more than 2 orders of magnitude less infectious than mature virions when subjected to titer determination on PS cells (12). The specific HA activity also increased in the same range, reaching a maximum at about 7 furin units. These properties were not affected by the addition of r-furin to mature virion preparations (data not shown).

Based on the evidence available from previous studies, it is likely that the observed increases in infectivity and HA activity upon prM cleavage reflect the activation of the fusogenic potential of the virus. To test this directly, a fusion-from-without assay was used (9, 32). In this experiment, untreated immature virions, immature virions that had first been activated with r-furin (see Materials and Methods), or mature virions were added to C6/36 cells and acidified to pH 5.5 and their ability to induce cell-cell fusion was assessed. Untreated immature virions, as expected, did not induce fusion (Fig. 6A), but after activation with r-furin, syncytium formation was observed (Fig. 6B), and the resulting polycaryocytes resembled those induced by mature virions (Fig. 6C).

In vitro cleavage of prM allows the envelope to undergo structural rearrangements. It has been suggested that prM inhibits fusion activity by physically blocking the low-pH-induced conversion of the E protein to its trimeric form, since these trimers are not formed in immature virions under the same conditions (1). To test whether, after in vitro activation, the E proteins in immature virions acquire the ability to trimerize at low pH, immature virions were activated and then treated under conditions shown previously to lead to a quantitative conversion of dimers to trimers in mature virions (1, 32). The activated virions were treated for 10 min at either pH

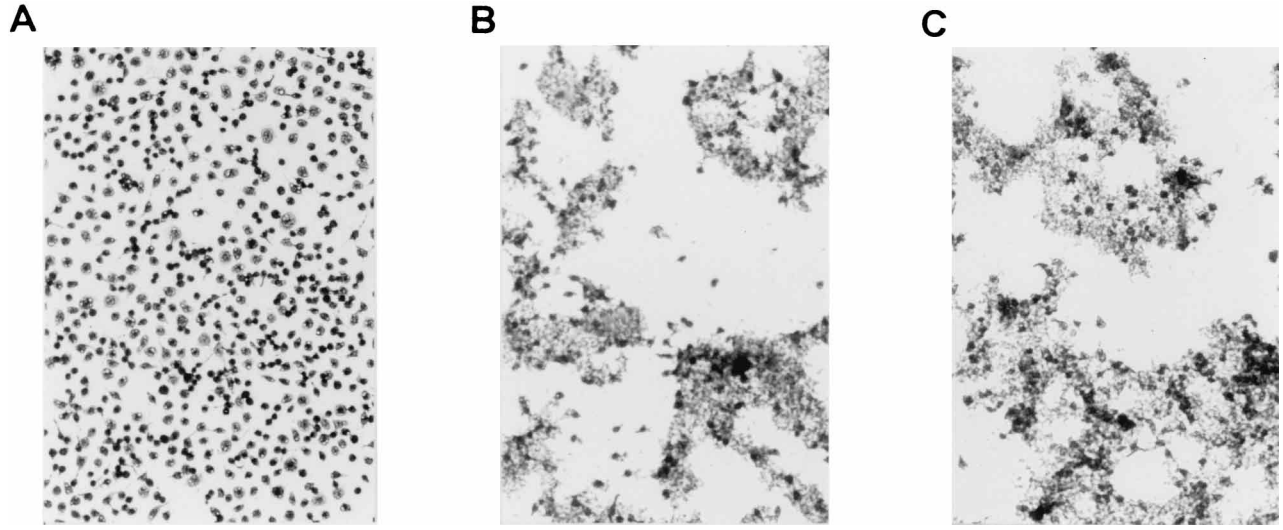

FIG. 6. Fusion activity of in vitro-activated immature virions. Different virus preparations were tested for their ability to induce fusion of C6/36 mosquito cells at pH 5.5. Shown are cells treated with immature virions without r-furin treatment (A), immature virions after in vitro activation with r-furin (B), and mature virions (C).

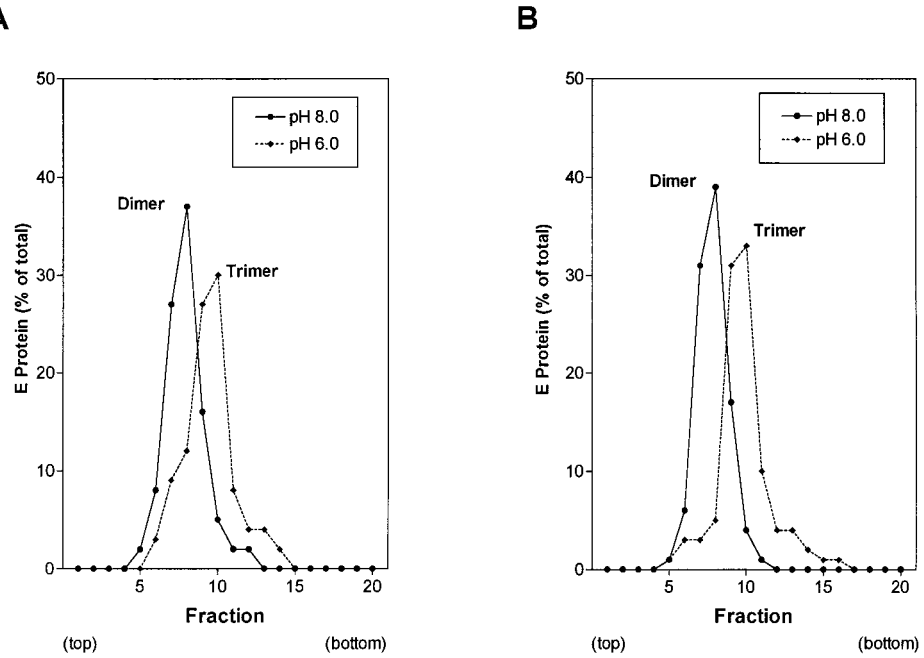

FIG. 7. Oligomeric rearrangements of E protein in in vitro-activated immature virions. Furin-treated immature virions (A) or mature virions (B) were solubilized with 0.5% Triton X-100 at pH 8.0 after a 10-min treatment at pH 6.0 or without low-pH treatment and analyzed by sedimentation on 7 to 20% sucrose gradients containing 0.1% Triton X-100. The positions of the dimer and trimer peaks are indicated.

8.0 or pH 6.0, back-neutralized to pH 8.0, and solubilized with 0.5% Triton X-100. The oligomeric state of the solubilized E proteins was then analyzed by sedimentation analysis on sucrose gradients (1).

Consistent with earlier observations (1, 12), solubilization of immature virus at pH 8.0 yielded a relatively broad sedimentation peak (data not shown) with a maximum at the position expected for the prM-E heterodimer (2). After treatment with r-furin (without subsequent acidification), essentially all of the solubilized E proteins sedimented as homodimers (Fig. 7A) resembling those of the native mature virus controls (Fig. 7B). A 10-min treatment at pH 6.0, however, induced a quantitative conversion of dimers to trimers in both the in vitro- and in vivo-activated virions (Fig. 7A and B), suggesting that cleavage of prM is sufficient for converting immature virions to their mature functional form.

DISCUSSION

It is a common strategy of enveloped viruses to synthesize components of the viral fusion machinery in an inactive precursor form which requires proteolytic cleavage for activation (16). Alphaviruses and flaviviruses use an intriguing variation on this theme, with a viral chaperone-like protein, rather than the fusion-active protein itself, playing the central role in proteolytic activation. The viruses of each of these genera have regular ordered envelope structures which undergo major rearrangements at low pH (1, 43), and in each case the presence of the uncleaved precursor protein appears to prevent these structural changes from occurring (1, 42).

Consistent with this hypothesis, we found that specific in vitro cleavage of prM in immature TBE virus led to the acquisition of properties normally associated with active mature virions—the ability of the envelope proteins to undergo low-pH-induced rearrangements and the ability of the virus to induce membrane fusion. These results further strengthen the argument that these events are related and that the reorganization of the envelope is a prerequisite for fusion. The increased specific infectivity and HA activity observed after prM cleavage are probably a direct consequence of the activation of the fusion potential of the virus, but it is still possible that the presence of prM also interferes with virus-cell interactions in other ways that have not yet been investigated.

The data presented here provide two lines of evidence that furin itself is important for flavivirus activation. First, a specific peptidyl chloromethyl ketone inhibitor which binds the active site of this enzyme was able to block cleavage of prM in infected BHK-21 cells. Similar results with furin-specific inhibitors have already implicated this enzyme in the proteolytic processing of the influenza virus HA protein (36), the human immunodeficiency virus type 1 (HIV-1) gp160 (10), the human parainfluenza virus F protein (26), and the cytomegalovirus gB protein (39), all of which have cleavage sites similar to that of prM. Second, virus particles secreted from LoVo cells, which lack functional furin (37, 38), contained only uncleaved prM and no detectable mature M. We therefore found no evidence of furin-independent cleavage in these cells. Similar studies with LoVo cells have suggested that influenza virus (14) and Newcastle disease virus (24) glycoproteins also require furin for activation whereas gp160 of HIV-1 can also be cleaved in LoVo cells by a different cellular protease (24).

The other major finding of the present study is that preexposure of the immature virion to low pH is required for cleavage with r-furin. After exposure, however, low pH is no longer required and prM can be cleaved equally well at acidic and neutral pH values. It therefore appears that an irreversible structural change in the prM-E complex is necessary to make the cleavage site adequately accessible to the enzyme. This change is probably manifested primarily in the prM protein itself, since studies with conformation-specific monoclonal antibodies have shown that the conformation of the E protein in immature virions remains essentially unchanged after low-pH

treatment (12, 32). Interestingly, the in vivo cleavage activation of some other viral glycoprotein precursors, e.g., gp160 of HIV-1 (47), HA_0 of influenza virus (25), and F_0 of Newcastle disease virus (48), can also be inhibited by acidotropic agents which raise the pH of the TGN. Since furin is believed to be responsible for this cleavage (at least in the last two cases), it can be speculated that low-pH-induced conformational changes in the precursor proteins might play a role in these activation events as well.

The apparently paradoxical role of acidic pH in flavivirus activation leaves some puzzling questions unresolved. Current evidence suggests that the lumen of the TGN and of post-Golgi vesicles, where furin is abundant and where flavivirus cleavage presumably takes place, is acidic (3, 33), with a sufficiently low pH to rapidly trigger irreversible structural changes in mature virions (12). If newly activated virions are immediately susceptible to low-pH inactivation, it is not clear how they manage to escape intact. One possibility is that the removed N-terminal "pr" segment, which has so far been found only outside of the cell (23, 27), might continue to play a protective role until the virus is actually released. Alternatively, the actual site of cleavage might turn out to be in an environment that is less acidic than the TGN. In this context, it is of interest that furin-mediated proteolytic cleavage of the Semliki Forest virus p62 protein (30) and of the HIV-1 gp160 protein (15) has been reported to occur after the virus has already left the TGN. More detailed mapping of the intracellular location of prM cleavage is necessary for an understanding of how activation and release are spatially and temporally coordinated. However, it is already now becoming evident that acidic pH plays a crucial role in both the entry and release phases of the flavivirus life cycle and that these relatively simple viruses have developed sophisticated mechanisms for exploiting the local environment of cellular compartments.

ACKNOWLEDGMENTS

We thank Martin Vey, Wolfgang Garten, and Hans-Dieter Klenk for generously providing the r-furin plasmid clone and the peptidyl-CMK inhibitor. We are also grateful to Jutta Ertl, Angela Dohnal, and Walter Holzer for excellent technical assistance.

Part of this work was supported by a grant from the International Human Frontier Science Program.

REFERENCES

1. **Allison, S. L., J. Schalich, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J. Virol. **69:**695–700.
2. **Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J. Virol. **69:**5816–5820.
3. **Anderson, R. G. W., and L. Orci.** 1988. A view of acidic intracellular compartments. J. Cell Biol. **106:**539–543.
4. **Bosshart, H., J. Humphrey, E. Deignan, J. Davidson, J. Drazba, L. Yuan, V. Oorschot, P. J. Peters, and J. S. Bonifacino.** 1994. The cytoplasmic domain mediates localization of furin to the *trans*-Golgi network en route to the endosomal/lysosomal system. J. Cell Biol. **126:**1157–1172.
5. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44:**649–688.
6. **Clarke, D. A., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7:**561–573.
7. **Denault, J.-B., and R. Leduc.** 1996. Furin/PACE/SPC1: a convertase involved in exocytic and endocytic processing of precursor proteins. FEBS Lett. **379:**113–116.
8. **Guirakhoo, F., R. A. Bolin, and J. T. Roehrig.** 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology **191:**921–931.
9. **Guirakhoo, F., F. X. Heinz, C. W. Mandl, H. Holzmann, and C. Kunz.** 1991. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. J. Gen. Virol. **72:**1323–1329.
10. **Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H.-D. Klenk, and W. Garten.** 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature **360:**358–361.
11. **Heinz, F. X., and C. Kunz.** 1981. Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus: comparison with other flaviviruses. J. Gen. Virol. **57:**263–274.
12. **Heinz, F. X., K. Stiasny, G. Püschner-Auer, H. Holzmann, S. L. Allison, C. W. Mandl, and C. Kunz.** 1994. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology **198:**109–117.
13. **Holzmann, H., K. Stiasny, M. Ecker, C. Kunz, and F. X. Heinz.** 1997. Characterization of monoclonal antibody-escape mutants of tick-borne encephalitis virus with reduced neuroinvasiveness in mice. J. Gen. Virol. **78:**31–37.
14. **Horimoto, T., K. Nakayama, S. P. Smeekens, and Y. Kawaoka.** 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J. Virol. **68:**6074–6078.
15. **Kantanen, M. L., P. Leinikki, and E. Kuismanen.** 1995. Endoproteolytic cleavage of HIV-gp160 envelope precursor occurs after exit from the trans-Golgi network (TGN). Arch. Virol. **140:**1441–1449.
16. **Klenk, H.-D., and W. Garten.** 1994. Activation cleavage of viral spike proteins by host proteases, p. 241–280. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80:**575–599.
18. **Lobigs, M., and H. Garoff.** 1990. Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein precursor p62. J. Virol. **64:**1233–1240.
19. **Mandl, C. W., F. X. Heinz, and C. Kunz.** 1988. Sequence of the structural proteins of tick-borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. Virology **166:**197–205.
20. **Molloy, S. S., P. A. Bresnahan, S. H. Leppla, K. R. Klimpel, and G. Thomas.** 1992. Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J. Biol. Chem. **267:**16396–16402.
21. **Molloy, S. S., L. Thomas, J. K. VanSlyke, P. E. Stenberg, and G. Thomas.** 1994. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. EMBO J. **13:**18–33.
22. **Monath, T. P., and F. X. Heinz.** 1996. Flaviviruses, p. 961–1034. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
23. **Murray, J. M., J. G. Aaskov, and P. J. Wright.** 1993. Processing of the dengue virus type 2 proteins prM and C-prM. J. Gen. Virol. **74:**175–182.
24. **Ohnishi, Y., T. Shioda, K. Nakayama, S. Iwata, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1994. A furin-defective cell line is able to process correctly the gp160 of human immunodeficiency virus type 1. J. Virol. **68:**4075–4079.
25. **Ohuchi, M., A. Cramer, M. Vey, R. Ohuchi, W. Garten, and H.-D. Klenk.** 1994. Rescue of vector-expressed fowl plague virus hemagglutinin in biologically active form by acidotropic agents and coexpressed M_2 protein. J. Virol. **68:**920–926.
26. **Ortmann, D., M. Ohuchi, H. Angliker, E. Shaw, W. Garten, and H.-D. Klenk.** 1994. Proteolytic cleavage of wild type and mutants of the F protein of human parainfluenza virus type 3 by two subtilisin-like endoproteases, furin and Kex2. J. Virol. **68:**2772–2776.
27. **Randolph, V. B., G. Winkler, and V. Stollar.** 1990. Acidotropic amines inhibit proteolytic processing of flavivirus prM protein. Virology **174:**450–458.
28. **Rice, C. M.** 1996. Flaviviridae: the viruses and their replication, p. 931–959. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
29. **Salminen, A., J. M. Wahlberg, M. Lobigs, P. Liljeström, and H. Garoff.** 1992. Membrane fusion process of Semliki Forest virus. II. Cleavage-dependent reorganization of the spike protein complex controls virus entry. J. Cell Biol. **116:**349–357.
30. **Sariola, M., J. Saraste, and E. Kuismanen.** 1995. Communication of post-Golgi elements with early endocytic pathway: regulation of endoproteolytic cleavage of Semliki Forest virus p62 precursor. J. Cell Sci. **108:**2465–2475.
31. **Schäfer, W., A. Stroh, S. Berghöfer, J. Seiler, M. Vey, M.-L. Kruse, H.-F. Kern, H.-D. Klenk, and W. Garten.** 1995. Two independent targeting signals in the cytoplasmic domain determine *trans*-Golgi network localization and endosomal trafficking of the proprotein convertase furin. EMBO J. **14:**2424–2435.
32. **Schalich, J., S. L. Allison, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. **70:**4549–4557.

33. **Seksek, O., J. Biwersi, and A. S. Verkman.** 1995. Direct measurement of *trans*-Golgi pH in living cells and regulation by second messengers. J. Biol. Chem. **270:**4967–4970.
34. **Shapiro, D., W. E. Brandt, and P. K. Russell.** 1972. Change involving a viral membrane glycoprotein during morphogenesis of group B arboviruses. Virology **50:**906–911.
35. **Smeekens, S. P.** 1993. Processing of protein precursors by a novel family of subtilisin-related mammalian endoproteases. Bio/Technology **11:**182–186.
36. **Stieneke-Gröber, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.-D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. **11:**2407–2414.
37. **Takahashi, S., K. Kasai, K. Hatsuzawa, N. Kitamura, Y. Misumi, Y. Ikehara, K. Murakami, and K. Nakayama.** 1993. A mutation of furin causes the lack of precursor-processing activity in human colon carcinoma LoVo cells. Biochem. Biophys. Res. Commun. **195:**1019–1026.
38. **Takahashi, S., T. Nakagawa, K. Kasai, T. Banno, S. J. Duguay, W. J. M. Van de Ven, K. Murakami, and K. Nakayama.** 1995. A second mutant allele of furin in the processing-incompetent cell line, LoVo. J. Biol. Chem. **270:** 26565–26569.
39. **Vey, M., W. Schäfer, S. Berghöfer, H.-D. Klenk, and W. Garten.** 1994. Maturation of the *trans*-Golgi network protease furin: compartmentalization of propeptide removal, substrate cleavage, and COOH-terminal truncation. J. Cell Biol. **127:**1829–1842.
40. **Vey, M., W. Schäfer, B. Reis, R. Ohuchi, W. Britt, W. Garten, H.-D. Klenk, and K. Radsak.** 1995. Proteolytic processing of human cytomegalovirus glycoprotein B (gpUL55) is mediated by the human endoprotease furin. Virology **206:**746–749.
41. **Vidricaire, G., J.-B. Denault, and R. Leduc.** 1993. Characterization of a secreted form of human furin endoprotease. Biochem. Biophys. Res. Commun. **195:**1011–1018.
42. **Wahlberg, J. M., W. A. M. Boere, and H. Garoff.** 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. J. Virol. **63:**4991–4997.
43. **Wahlberg, J. M., R. Bron, J. Wilschut, and H. Garoff.** 1992. Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. J. Virol. **66:**7309–7318.
44. **Watson, D. G., J. M. Moehring, and T. J. Moehring.** 1991. A mutant CHO-K1 strain with resistance to *Pseudomonas* exotoxin A and alphaviruses fails to cleave Sindbis virus glycoprotein PE2. J. Virol. **65:**2332–2339.
45. **Wengler, G., and G. Wengler.** 1989. Cell-associated West Nile flavivirus is covered with E + Pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. J. Virol. **63:**2521–2526.
46. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
47. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
48. **Yoshida, T., S. Takao, K. Kiyotani, and T. Sakaguchi.** 1989. Endoproteolytic activation of Newcastle disease virus fusion proteins requires an intracellular acidic environment. Virology **170:**571–574.